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Docket Number: 1328-005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of
PADGETT et al.
Serial No.: 10/684,134
Filed: October 10, 2003
For: Polynucleotide Sequence Variants

Examiner: Amanda M. SHAW
Group Art Unit: 1634

I hereby certify that this correspondence is being deposited with the United States Postal service with sufficient postage for first class mail in a package addressed to Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on August 13, 2007

by Wayne Fitzmaurice
Wayne P. Fitzmaurice
Reg. No. 58,274

DECLARATION UNDER RULE 132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

August 13, 2007

Dear Sir:

I, Hal S. Padgett, do hereby declare and state:

1. I received a B.S. in Life Sciences in 1988 from the University of Missouri at Rolla and a Ph.D. in Molecular Microbiology and Microbial Pathogenesis in 1996 from Washington University in Saint Louis, Missouri. I joined Large Scale Biology Corporation in 1998, and am presently Scientific Director. For the past nine years my responsibilities have involved viral vector development and molecular evolution programs at Large Scale Biology Corporation.

2. I am a named inventor of the subject matter that is claimed and for which a patent

is sought on the invention entitled POLYNUCLEOTIDE SEQUENCE VARIANTS.

3. The methods described in this application yield products that are surprisingly and unexpectedly different from that which is obtained simply from treatment of heteroduplexes with a DNA repair system.

4. The following data serves to further illustrate the results obtained in experiments of the type described in Examples 3, 4, 6, and 10 in the specification.

5. The series of experiments described in Examples 3 and 4 of the specification were performed to demonstrate that the Genetic Reassortment by Mismatch Resolution (GRAMMR) method efficiently creates shuffled gene sequences from linear heteroduplex molecules. Although the results of this experiment have already been presented in the specification, the results are shown in greater detail in Figure A as a series of graphical DNA alignments of the shuffled progeny genes with the parent genes to further illustrate the nature of the output from the claimed process and to contrast those results with results obtained from exposing heteroduplex DNA to a DNA repair system.

In this experiment, linear heteroduplex molecules were created by annealing two single-stranded DNAs of opposite strandedness generated by PCR from the two different parent genes described in Example 3 of the application. A sample of this heteroduplex DNA preparation was incubated in the presence of the GRAMMR reaction components, as described in Example 3, prior to cloning and sequencing. The negative control for the experiment consisted of a sample in which the GRAMMR reaction components were omitted, but was otherwise treated in parallel to the GRAMMR-treated heteroduplex sample. This negative control was included to measure the background level of sequence recombination that occurs when the non-GRAMMR-treated heteroduplex is exposed to a DNA repair system upon introduction into *E. coli* cells in a downstream processing step.

Figure A shows a graphical representation of the DNA sequences of randomly chosen output molecules from the experiment. The group of sequences from the negative control reaction is depicted in the top panel of Figure A and the group of sequences derived from the CEL I treated samples are shown in the bottom panel of Figure A. In each figure each sequence is depicted in two ways: a 'nucleotide view' and a 'reassortment view' as shown. In the 'nucleotide view', gray represents nucleotides that are in common between the two parent molecules and dark blue or green represents nucleotides that are specific to one or the other parent. Light blue and red represent mutations that may occasionally arise, usually as a result of PCR amplification. As one follows the representation of a particular sequence across the panel, one can see the sequence switching between dark blue and green representing information transfer events and corresponding sequence reassortment. The results are easier to visualize in bulk with the representation of the same sequences shown at the bottom portion of each figure panel. In this 'reassortment view' the dots show the midpoint between areas of the sequence that have switched from one parent sequence to the other (blue to green) or vice versa. Each of these is analogous to a recombinational crossover event.

The overall results of this shuffling experiment are described in Example 4 of the application, and a representative set of the data are shown here. Only two of 10 clones derived from the negative control showed sequence recombination, with each of those having only a single crossover event (Figure A, top panel). Relatively infrequent crossover events among the negative controls are typical in our hands and are presumed to be caused either by the effects of the *E. coli* DNA repair system as described previously (1, 2, 3, 4, 9, 10) or, because the DNAs were PCR amplified prior to cloning, by 'jumping PCR' as described by Paabo (7).

In contrast to the negative controls, 100% of the GRAMMR-treated samples were shuffled (Figure A, bottom panel). Additionally, these samples displayed an average of 5 crossovers per clone which is equivalent to roughly nine per kilobase of heteroduplex region in the substrate. Both the percentage of shuffled clones and the amount of sequence reassortment are far higher in the GRAMMR-treated samples than in the DNA repair system-treated samples (negative controls).

6. Another experiment is described to illustrate the use of the GRAMMR method and to highlight the differences between results from the GRAMMR process and results from the use of a DNA repair system to treat heteroduplexes. This experiment was performed as previously described in Example 6 of the application.

The negative control for this experiment received buffer instead CEL I, but was otherwise identical to the CEL I-treated experimental samples. This control reaction was performed to monitor the background level of sequence reassortment that occurs at a low frequency when heteroduplex substrates are introduced into *E. coli* in a downstream processing step and are presumably directly acted upon by the DNA repair system of the host cell.

After one hour of incubation of all reactions at 25 degrees centigrade, one microliter aliquots of each were used to transform competent DH5-alpha *E. coli* that were then plated on solid medium plates. A number of resulting colonies from the agar plates corresponding to each reaction were randomly picked and inoculated to liquid cultures. Plasmid DNA was extracted from each of the cultures followed by DNA sequence analysis of the complete GFP gene in each clone. The nucleotide sequences of these clones were analyzed by comparison to the two original parent GFP genes encoded by pBSWTGFP and pBSC3GFP. The results of this analysis are displayed in Figure B.

Figure B shows a graphic representation of the DNA sequences of output molecules from the experiment. The group of sequences from the negative control reaction is depicted in the top panel of Figure B and the group of sequences derived from the CEL I treated samples are shown in the bottom panel of Figure B. In each panel each sequence is depicted in two ways: a ‘nucleotide view’ and a ‘reassortment view’ as shown. In the ‘nucleotide view’, gray represents nucleotides that are in common between the two parent molecules and dark blue or green represents nucleotides that are specific to one or the other parent. Light blue and red represent mutations or sequencing artifacts that are occasionally observed. As one follows the representation of a particular sequence across the panel, one can see the sequence switching between dark blue and green representing information transfer events and corresponding sequence reassortment. The results are easier to visualize in bulk with the representation of the same sequences shown at the bottom of each figure panel. In this 'reassortment view' the dots show the midpoint between areas of the sequence that have switched from one parent sequence to the other (blue to green) or vice versa. Each of these is analogous to a recombinational crossover event.

Figure B, top panel depicts the results from the negative control showing only one recombined clone in ten. This is a typical result in which the few recombinants that are observed in the negative controls show only a single or double crossover. Such results presumably reflect the level of sequence reassortment that is caused by the action of the cell's DNA repair system on the heteroduplex substrate and are consistent to what has been reported previously (1, 2, 3, 4, 9, 10).

In contrast to the negative control samples, molecular clones derived from heteroduplex DNAs exposed to complete GRAMMR reactions that included the CEL I mismatch endonuclease enzyme were extensively shuffled by only a single round of the GRAMMR process. As is shown in Figure B, bottom panel, the number of progeny clones bearing shuffled sequences is much higher in the CEL I treated samples than in the negative controls containing all reaction components but with CEL I omitted (eight of ten samples vs. one of ten, respectively).

From these data, it is evident that the GRAMMR process yields a reaction product that differs from what is obtained by simple exposure of a heteroduplex DNA to a DNA repair system.

7. The following experiment was described in Example 10 of the application and was performed to demonstrate that the claimed process is capable of reassortment of divergent gene sequences. In addition, the experiment provides further support for the ability of the method to create shuffled gene libraries from treatment of linear heteroduplexes as claimed in the present application. This experiment also highlights the

contrast between the results obtained from the GRAMMR method and those following exposure of the heteroduplex to a DNA repair system.

The sequences being shuffled are homologous genes that share about 75% nucleotide identity. A linear heteroduplex DNA preparation was made and either treated using the GRAMMR process or used as a negative control by omitting the CEL I mismatch endonuclease enzyme from the reaction as described in Example 10. The results of this experiment are shown in Figure C.

Figure C shows a graphic representation of the DNA sequences of randomly chosen output molecules from the experiment. The group of sequences from the negative control reaction is depicted in the top panel of Figure C and the group of sequences derived from the CEL I treated samples are shown in the bottom panel of Figure C. In each panel each sequence is depicted in two ways: a 'nucleotide view' and a 'reassortment view' as shown. In the 'nucleotide view', gray represents nucleotides that are in common between the two parent molecules and dark blue or green represents nucleotides that are specific to one or the other parent. Light blue and red represent mutations that may occasionally arise, presumably as a result of PCR amplification. As one follows the representation of a particular sequence across the panel, one can see the sequence switching between dark blue and green reflecting the occurrence of information transfer events and corresponding sequence reassortment. The results are easier to visualize in bulk with the representation of the same sequences shown at the bottom of each figure panel. In this 'reassortment view' the dots show the midpoint between areas of the sequence that have switched from one parent sequence to the other (blue to green) or vice versa. Each of these is analogous to a recombinational crossover event.

The overall results of this experiment are described in Example 10 of the application and are summarized here. None of the seven negative control clones shown in the top panel of Figure C showed any sequence recombination.

In contrast, seven out of eight of the GRAMMR-treated samples had been shuffled (Figure C, bottom panel) with an average of about 2.5 crossovers per clone.

The results of this experiment demonstrate the contrast between the effects of treating heteroduplex DNA substrates with the claimed invention versus the effects of treatment of the heteroduplex with a DNA repair system.

Additional experiments are described here that are not included in the examples of the application since they had not been performed at that time. However, these examples provide additional support for the difference between the claimed invention and the use of a DNA repair system to create shuffled DNA molecules. The negative controls are treated the same in each case, and are therefore useful for assessing the level of sequence reassortment caused by treatment with a DNA repair system.

8. Another experiment is described to further illustrate the use of the GRAMMR method and to highlight the differences between results from the GRAMMR process and results from the use of a DNA repair system to treat heteroduplexes.

This experiment was performed by the method described in Example 6 of the application. Heteroduplex DNAs used as substrates in the experiment were generated by restriction enzyme linearizing the parental plasmid pGW-U1MP with Stu I and the parental plasmid pGW-ToMVMP with Sma I followed by spin column purification of the linearized plasmids, mixing and heat dissociation of the complementary strands and annealing of the resulting single stranded DNAs to one another to form duplexed DNAs. Because these parental plasmids differed in the approximately 800 base pair movement protein (MP) gene regions, but not the rest of the plasmid, the mismatch-containing region of DNA was limited to the MP gene. The MP genes share about 75% nucleotide identity.

The DNA samples containing heteroduplex DNAs were used as substrate in Genetic Reassortment by Mismatch Resolution (GRAMMR) reactions in which a replicate series of 10 microliter reactions was prepared with each containing 1 microliter of the heteroduplex substrate, 1X NEB ligase buffer, 50 mM KCl, 0.5 mM each dNTP, 2.0 units *E. coli* DNA ligase (NEB), and 1.0 unit of T4 DNA polymerase, in the presence of various concentrations of CEL I. The CEL I was from a cloned preparation and the amount used varied from 0.33 microliters at the highest, through a series of four, 3-fold serial dilutions. The only difference among individual reactions in this experiment was the amount of CEL I added.

The negative control for this experiment consisted of a final replicate in the series that received buffer instead CEL I, but was otherwise identical to the above series of experimental samples. This control reaction was performed to monitor the background level of sequence reassortment that has been reported to occur at a low frequency when heteroduplex substrates are introduced into *E. coli* and are directly acted upon by the DNA repair system of the host cell (1, 2, 3, 4, 9, 10).

After one hour of incubation of all reactions at 25 degrees centigrade and 30 minutes on ice, one microliter aliquots of each were used to transform competent DH5-alpha strain *E. coli* that were then plated on solid medium plates. A number of resulting colonies from the agar plates corresponding to each reaction were randomly picked and inoculated to liquid cultures. Plasmid DNA was extracted from each of the cultures followed by DNA sequence analysis of the complete movement protein gene in each clone. The nucleotide sequences of these clones were analyzed by comparison to the two original parent genes encoded by pGW-U1MP and pGW-ToMVMP. The results of this analysis are displayed in Figure D.

Figure D shows a graphic representation of the DNA sequences of output molecules from the experiment. The group of sequences from the negative control reaction is depicted in the top panel of Figure D and the group of sequences derived from the CEL I treated samples are shown in the bottom panel of Figure D. In each figure each sequence is depicted in two ways: a 'nucleotide view' and a 'reassortment view' as shown. In the 'nucleotide view', gray represents nucleotides that are in common between the two parent molecules and dark blue or yellow represents nucleotides that are specific to one or the other parent. Light blue represents mutations or sequencing artifacts that are occasionally observed. As one follows the representation of a particular sequence across the panel, one can see the sequence switching between dark blue and yellow representing information transfer events and corresponding sequence reassortment. The results are easier to visualize in bulk with the representation of the same sequences shown at the bottom of each figure panel. In this 'reassortment view' the dots show the midpoint between areas of the sequence that have switched from one parent sequence to the other (blue to yellow) or vice versa. Each of these is analogous to a recombinational crossover event.

The negative control sample in which the heteroduplexes were only exposed to the cellular repair system gave zero recombined clones out of 12 that were sequenced. Eight of these are depicted in Figure D, top panel. In sharp contrast, molecular clones derived from heteroduplex DNAs exposed to complete GRAMMR reactions that included the CEL I mismatch endonuclease enzyme were extensively shuffled in the single round of the process with six of eight clones being shuffled (Figure D, bottom panel). In addition, the frequency of sequence reassortment events caused by the GRAMMR process, with an average of more than eight in each clone, is very high.

From these data, it is evident that the GRAMMR process yields a reaction product that differs from what is obtained by simple exposure of a heteroduplex DNA to a DNA repair system.

9. Another experiment is described to further illustrate the use of the GRAMMR method and to highlight the differences between results from the GRAMMR process and results from the use of a DNA repair system to treat heteroduplexes.

This experiment was performed by the method described in Example 6 of the application. Heteroduplex DNAs used as substrates in the experiment were generated by restriction enzyme digestion of the parental plasmid pBSWTGFP with NgoM-IV and the parental plasmid pBSC3BFP, a blue fluorescent variant of pBSC3GFP, with KpnI followed by spin column purification of the linearized plasmids, mixing and heat dissociation of the complementary strands and annealing of the resulting single stranded DNAs to one another to form duplexed DNAs.

The DNA samples containing heteroduplex DNAs were used as substrate in Genetic Reassortment by Mismatch Resolution (GRAMMR) reactions in which a replicate series of 10 microliter reactions was prepared with each containing 1 microliter of the heteroduplex substrate, 1X NEB ligase buffer, 0.5 mM each dNTP, 2.0 units *E. coli* DNA ligase (NEB), and 1.0 unit of T4 DNA polymerase, in the presence of various concentrations of CEL I. The CEL I was from a cloned preparation and the amount used varied from 0.11 microliters at the highest, through a series of four, 3-fold serial dilutions. The only difference among individual reactions in this experiment was the amount of CEL I added.

The negative control for this experiment consisted of a final replicate in the series that received buffer instead CEL I, but was otherwise identical to the above series of experimental samples. This control reaction was performed to monitor the background level of sequence reassortment that has been reported to occur at a low frequency when heteroduplex substrates are introduced into *E. coli* in a downstream processing step and are directly acted upon by the DNA repair system of the host cell (1, 2, 3, 4, 9, 10).

After one hour of incubation of all reactions at 25 degrees centigrade, one microliter aliquots of each were used to transform competent XL1-Blue strain *E. coli* that were then plated on solid medium plates. A number of resulting colonies from the agar plates corresponding to each reaction were randomly picked and inoculated to liquid cultures. Plasmid DNA was extracted from each of the cultures followed by DNA sequence analysis of the complete GFP gene in each clone. The nucleotide sequences of these clones were analyzed by comparison to the two original parent genes encoded by pBSWTGFP and pBSC3BFP. The results of this analysis are displayed in Figure E.

Figure E shows a graphic representation of the DNA sequences of output molecules from the experiment. The group of sequences from the negative control reaction is depicted in the top panel of Figure E and the group of sequences derived from the CEL I treated samples are shown in the bottom panel of Figure E. In each figure each sequence is depicted in two ways: a 'nucleotide view' and a 'reassortment view' as shown. In the 'nucleotide view', gray represents nucleotides that are in common between the two parent molecules and dark blue or green represents nucleotides that are specific to one or the other parent. Light blue represents mutations or sequencing artifacts that are occasionally observed. As one follows the representation of a particular sequence across the panel, one can see the sequence switching between dark blue and green representing information transfer events and corresponding sequence reassortment. The results are easier to visualize in bulk with the representation of the same sequences shown at the bottom of each figure panel. In this 'reassortment view' the dots show the midpoint between areas of the sequence that have switched from one parent sequence to the other (blue to green) or vice versa. Each of these is analogous to a recombinational crossover event.

The negative control sample in which the heteroduplexes were only exposed to the cellular repair system gave zero recombined clones out of 14 that were sequenced. Ten of these are depicted in Figure E, top panel. In sharp contrast, molecular clones derived from heteroduplex DNAs exposed to complete GRAMMR reactions that included the CEL I mismatch endonuclease enzyme were extensively shuffled in the single round of the process with nine of 10 clones being shuffled (Figure E, bottom panel). In addition, the frequency of sequence reassortment events caused by the GRAMMR process, with an average of more than nine in each clone, is very high.

From these data, it is evident that the GRAMMR process yields a reaction product that is dramatically different from what is obtained by simple exposure of a heteroduplex DNA to a DNA repair system.

10. From these data, it is my opinion that the GRAMMR process yields shuffled molecular products that are different from those obtained from simply exposing heteroduplexes to a DNA repair system. The output polynucleotides from the negative controls in the accompanying figures result from exposure of heteroduplexes to a DNA repair system, as has already been amply described in the literature (1, 2, 3, 4, 9, 10). These controls therefore serve as a valid measure of the effects of a DNA repair system on the heteroduplex. These controls consistently show only low levels of sequence reassortment (an occasional recombined molecule, typically with only one crossover point) which is consistent with what has been reported previously when heteroduplexes with multiple mismatches were exposed to DNA repair systems either *in vivo* (1, 2, 3, 4, 9, 10) or *in vitro* (5, 6). DNA repair systems have been observed to perform similarly *in vitro* to the way they do *in vivo* (5, 8).

In marked contrast with results of the negative controls, the current invention produces very high frequencies of shuffled clones, usually with a high density of sequence reassortment events. If the GRAMMR shuffling method we describe were simply equivalent to a DNA repair system, it would be expected that similar results would be obtained from the claimed invention and from the negative controls. However, the results we get from the two are dramatically different, highlighting the contrast between the method of our invention and treatment with a DNA repair system.

These results are particularly relevant to Claims 1, 2, 4-12, 42 and 43 of the application.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information are believed to be true, and that the statements were made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, and patent issuing thereon, and patent to which this verified statement is directed.

August 13, 2007
Date

Hal S. Padgett, Ph.D.

Attachments:

- Reference Listing
- Figures A-E

- **References:**

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4. Fishel, R. and R. Kolodner, *Gene conversion in Escherichia coli: the recF pathway for resolution of heteroduplex DNA.* J Bacteriol, 1989. **171**(6): p. 3046-52.
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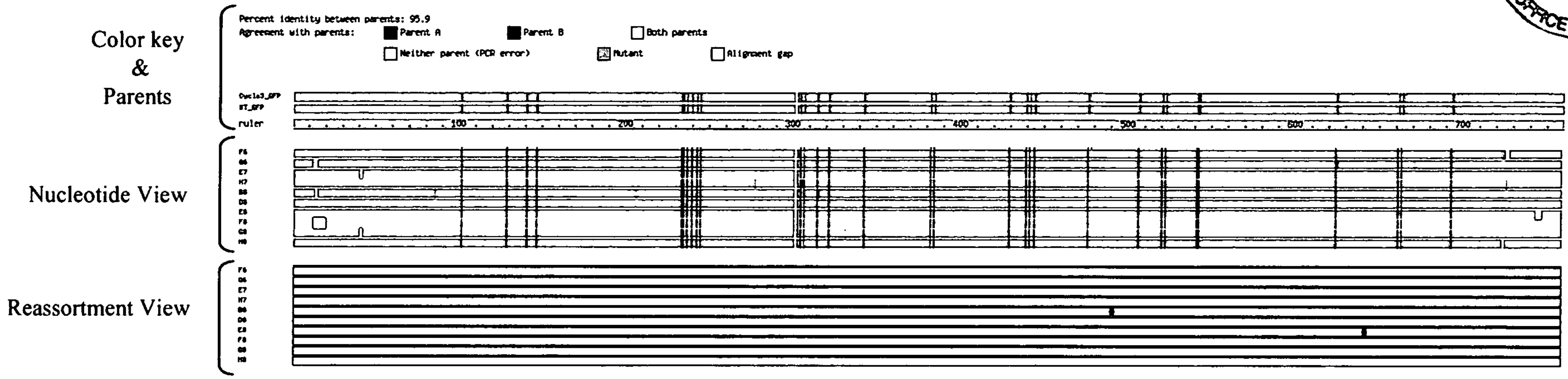
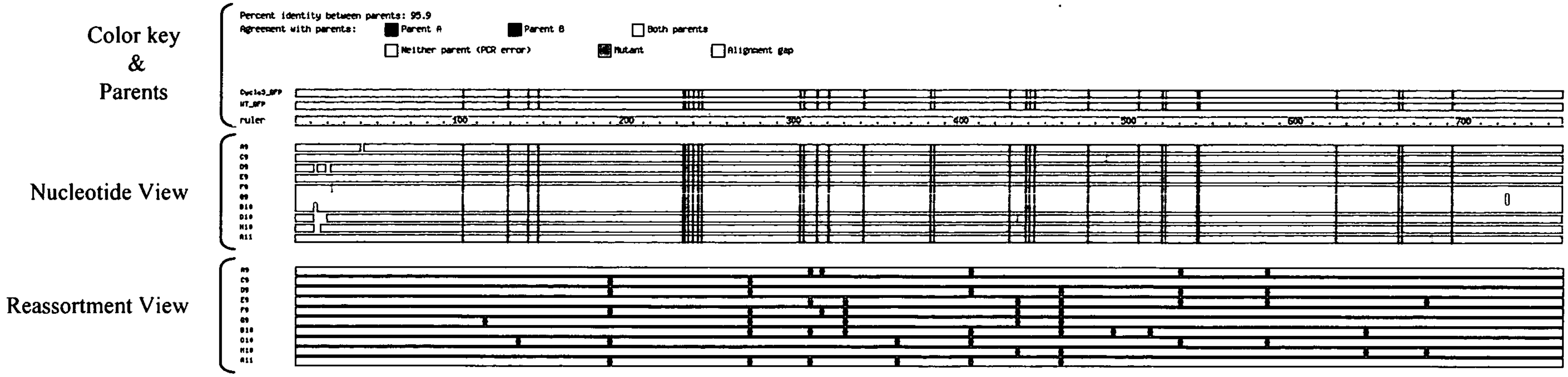
Figure A

No GRAMMR Treatment - Negative Control



(CEL I omitted from GRAMMR reaction)
Two Crossovers in 10 Progeny

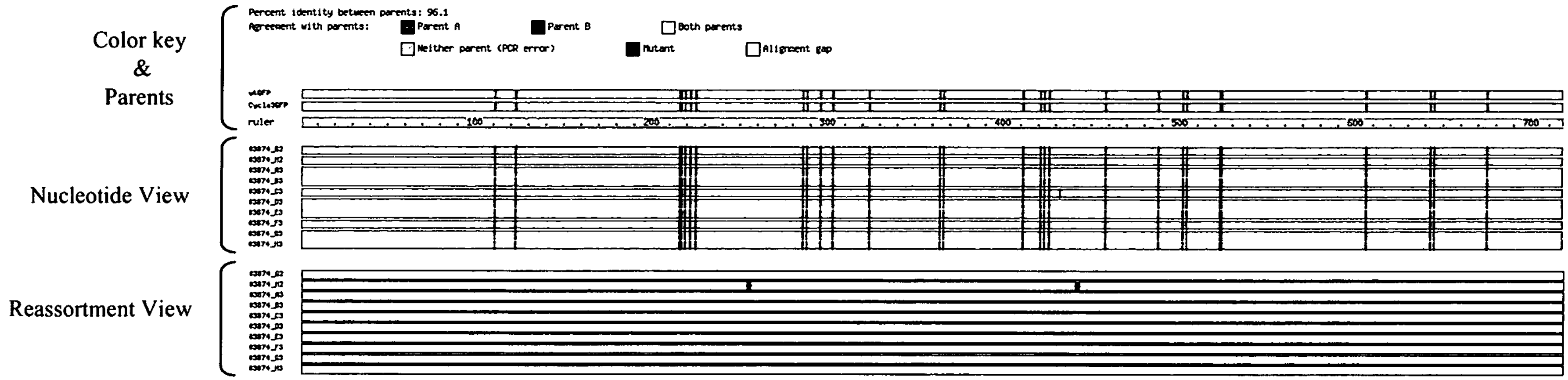
GRAMMR Treatment



55 Crossovers in 10 Progeny
(5/gene; 9/kilobase)

Figure B

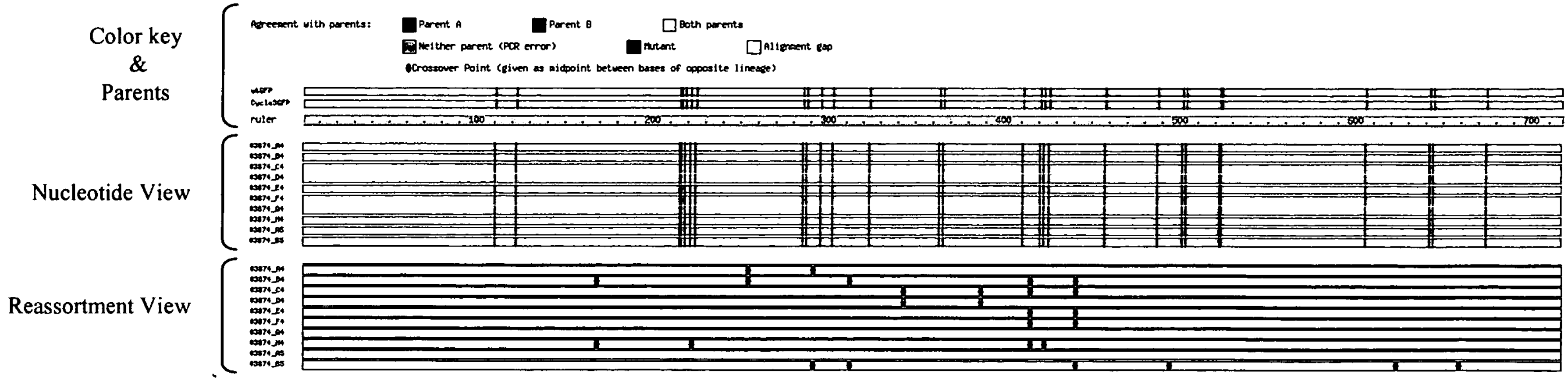
No GRAMMR Treatment - Negative Control



(CEL I omitted from GRAMMR reaction)

1 shuffled clone in 10 Progeny

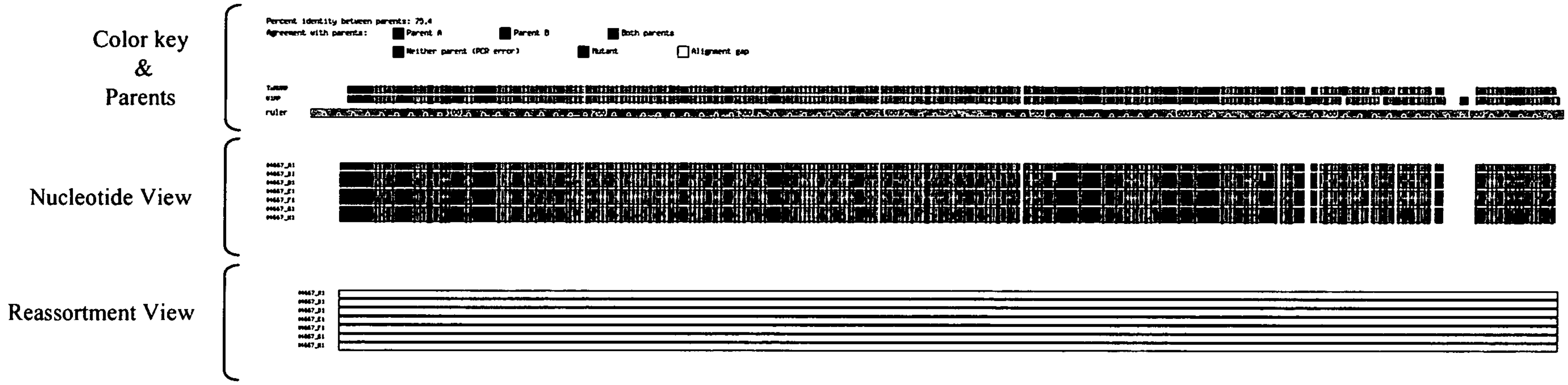
GRAMMR Treatment



27 Crossovers (2.7/gene; >4/kilobase)

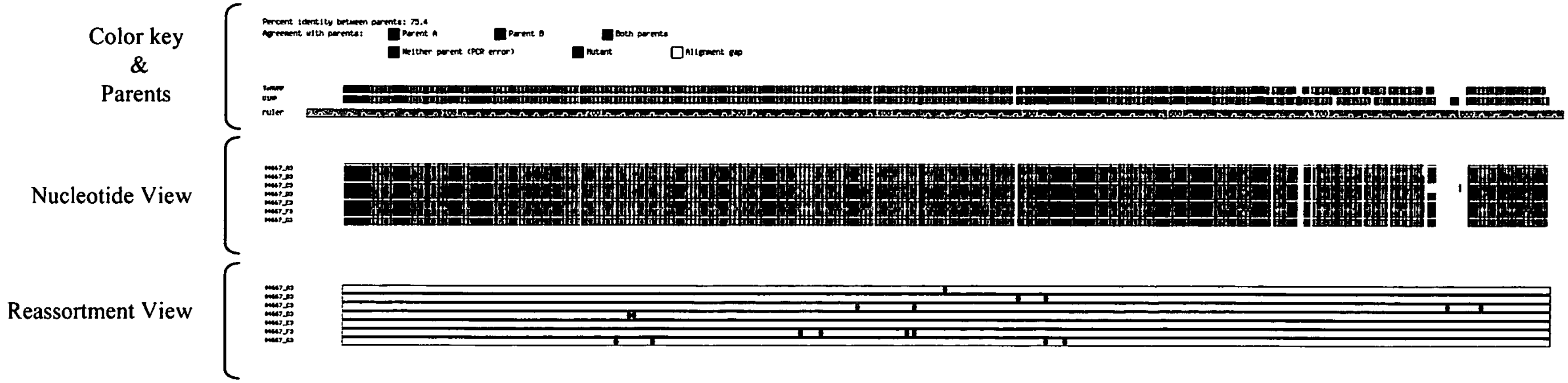
Figure C

No GRAMMR Treatment - Negative Control



(CEL I omitted from GRAMMR reaction)
Zero Crossovers in 8 Progeny

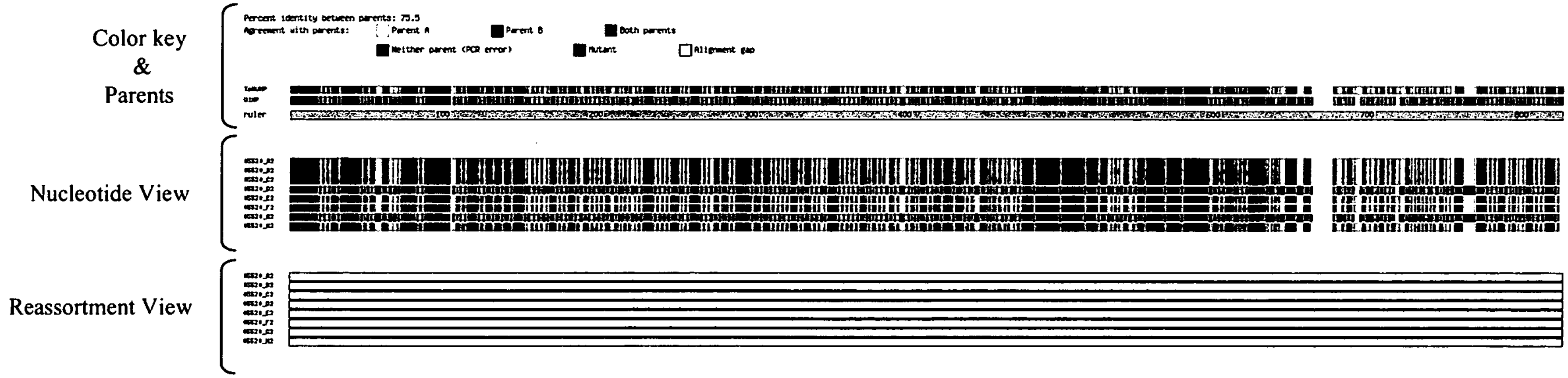
GRAMMR Treatment



21 Crossovers in 8 Progeny
(>2.5/gene; >3/kilobase)

Figure D

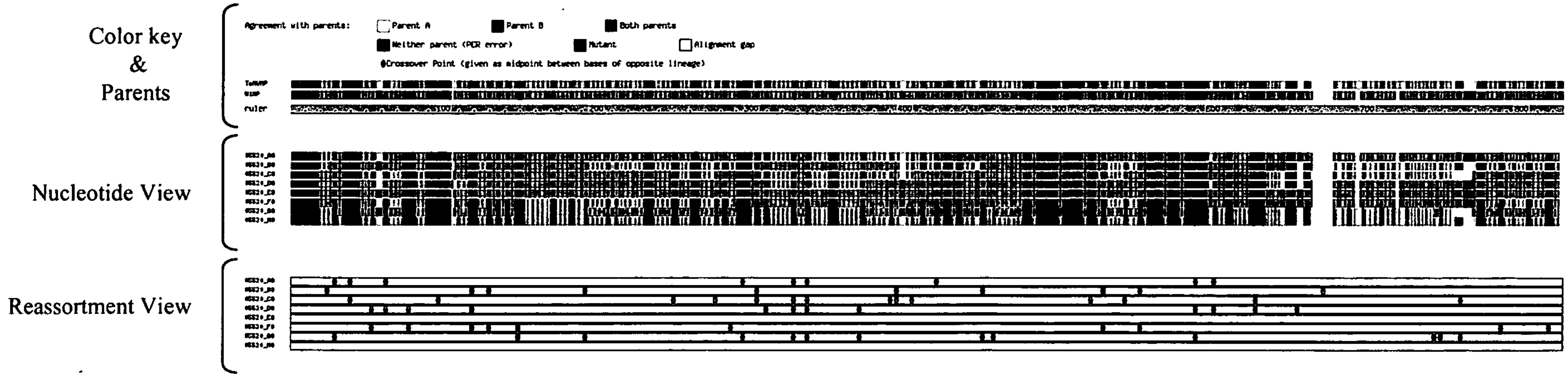
No GRAMMR Treatment - Negative Control



(CEL I omitted from GRAMMR reaction)

Zero Crossovers in 8 Progeny

GRAMMR Treatment

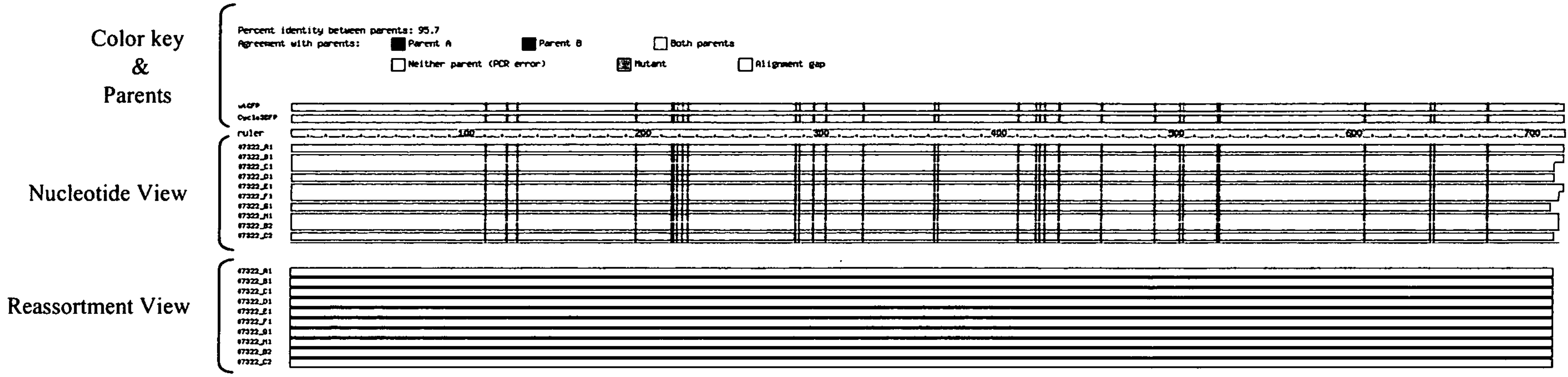


68 Crossovers in 8 Progeny

(>8/gene; >10/kilobase)

Figure E

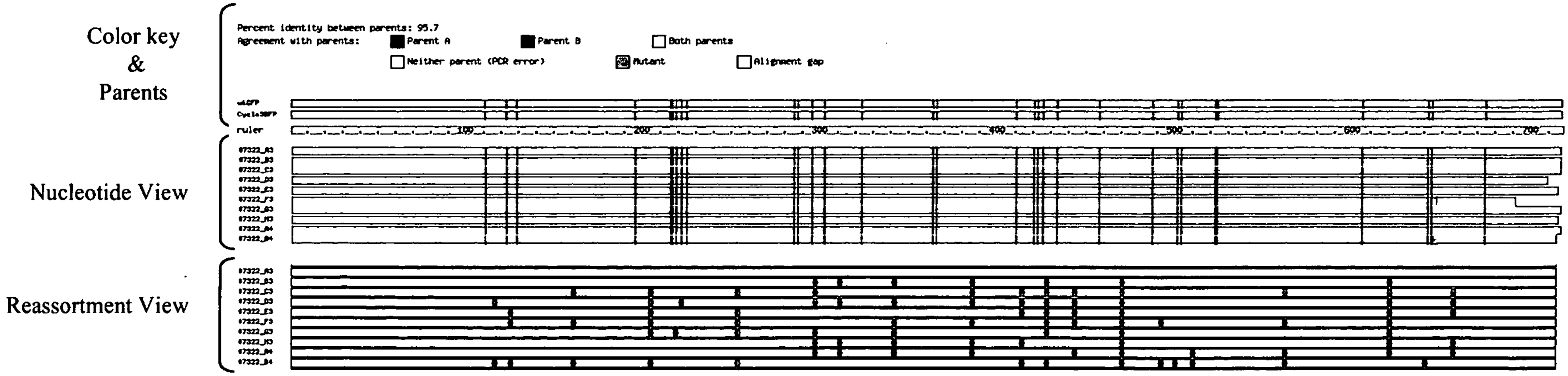
No GRAMMR Treatment - Negative Control



(CEL I omitted from GRAMMR reaction)

Zero Crossovers in 10 Progeny

GRAMMR Treatment



92 Crossovers (>9/gene; >12/kilobase)